PCT

WORLD INTELLECTUAL PROPERTY ORGANIZATION
International Bureau

INTERNATIONAL APPLICATION PUBLISHED UNDER THE PATENT COOPERATION TREATY (PCT)

(51) International Patent Classification 6 :
C12N 15/17, 1/06, 15/62

A1

(11) International Publication Number: WO 99/33988

(43) International Publication Date: 8 July 1999 (08.07.99)

(21) International Application Number: PCT/KR98/00464

(22) International Filing Date: 24 December 1998 (24.12.98)

(30) Priority Data:
1997/77163 29 December 1997 (29.12.97) KR

(71) Applicant *(for all designated States except US)*: CHONG KUN DANG CORPORATION [KR/KR]; 410, Shindorim-dong, Guro-gu, Seoul 152-070 (KR).

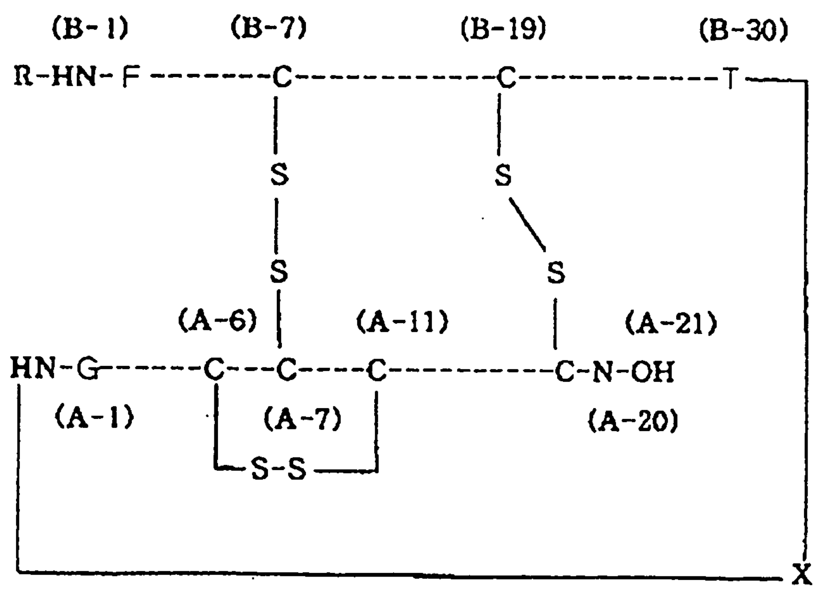
(72) Inventors; and
(75) Inventors/Applicants *(for US only)*: HONG, Chung-Il [US/US]; 100 Hunt Club Circle, East Amherst, NY 14051-1870 (US). KIM, Jung-Woo [KR/KR]; 925-7, Hwagog-dong, Kangseo-gu, Seoul 157-010 (KR). LEE, Wang-Sik [KR/KR]; 3-806 Misong Apartment, Apgujeong-dong, Kangnam-gu, Seoul 135-10 (KR). KIM, Chang-Kyu [KR/KR]; 9-301 Lucky Apartment, Sihung-dong, Kumchun-gu, Seoul 153-030 (KR). KIM, Yong-In [KR/KR]; 107-510 Hanjin Apartment, Kwangmyung-dong, Kwangmyung, Kyunggi-do 423-010 (KR). PHEU, Je-Nie [KR/KR]; 1008-602 Jookong Apartment, Haan 4-dong, Kwangmyung, Kyunggi-do 423-064 (KR). SHIN, Jeong-Woo [KR/KR]; 1513-202 Jookong Apartment, Shunbu-dong, Ansan, Kyunggi-do 425-140 (KR). OH, Sung-Jin [KR/KR]; 279-408 Jookong Apartment, Wonmun-dong, Kwachon, Kyunggi-do 427-030 (KR).

(74) Agent: LEE, Han-Young; Seowon Building, 8th floor, 1675-1, Seocho-dong, Seocho-gu, Seoul 137-070 (KR).

(81) Designated States: AL, AM, AT, AU, AZ, BA, BB, BG, BR, BY, CA, CH, CN, CU, CZ, DE, DK, EE, ES, FI, GB, GE, GH, GM, HR, HU, ID, IL, IS, JP, KE, KG, KP, KR, KZ, LC, LK, LR, LS, LT, LU, LV, MD, MG, MK, MN, MW, MX, NO, NZ, PL, PT, RO, RU, SD, SE, SG, SI, SK, SL, TJ, TM, TR, TT, UA, UG, US, UZ, VN, YU, ZW, ARIPO patent (GH, GM, KE, LS, MW, SD, SZ, UG, ZW), Eurasian patent (AM, AZ, BY, KG, KZ, MD, RU, TJ, TM), European patent (AT, BE, CH, CY, DE, DK, ES, FI, FR, GB, GR, IE, IT, LU, MC, NL, PT, SE), OAPI patent (BF, BJ, CF, CG, CI, CM, GA, GN, GW, ML, MR, NE, SN, TD, TG).

Published
With international search report.

(54) Title: A PROCESS FOR PREPARING HUMAN PROINSULIN



(I)

(57) Abstract

The present invention relates to a process for preparing human proinsulin which is represented as chemical formula (I) wherein R is an amino acid residue or a peptide which is degradable enzymatically or chemically; and X is a linkage of an amino group of A-1 in insulin A chain and a carboxyl group of B-30 in insulin B chain which can be separated from the A chain or the B chain enzymatically or chemically, provided that a region from A-1 to A-21 is the insulin A chain and a region from B-1 to B-30 is the insulin B chain. In accordance with the present invention, human recombinant insulin precursor can be easily manufactured with a good reproducibility, since dissolution, sulfonation, concentration, desalting and purification are remarkably simplified, while increasing the yield of refolding reaction.

FOR THE PURPOSES OF INFORMATION ONLY

Codes used to identify States party to the PCT on the front pages of pamphlets publishing international applications under the PCT.

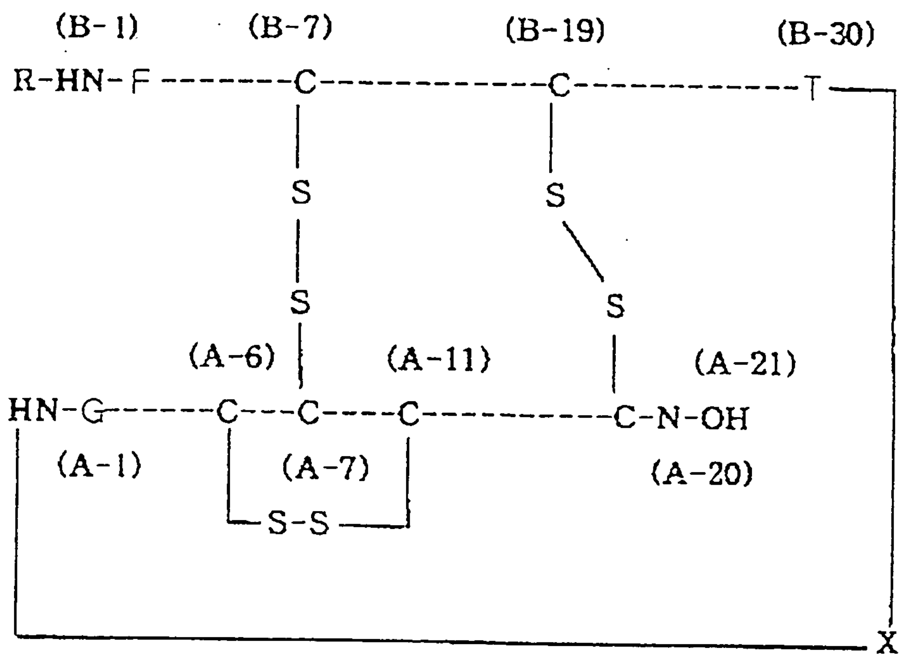
AL	Albania	ES	Spain	LS	Lesotho	SI	Slovenia
AM	Armenia	FI	Finland	LT	Lithuania	SK	Slovakia
AT	Austria	FR	France	LU	Luxembourg	SN	Senegal
AU	Australia	GA	Gabon	LV	Latvia	SZ	Swaziland
AZ	Azerbaijan	GB	United Kingdom	MC	Monaco	TD	Chad
BA	Bosnia and Herzegovina	GE	Georgia	MD	Republic of Moldova	TG	Togo
BB	Barbados	GH	Ghana	MG	Madagascar	TJ	Tajikistan
BE	Belgium	GN	Guinea	MK	The former Yugoslav Republic of Macedonia	TM	Turkmenistan
BF	Burkina Faso	GR	Greece			TR	Turkey
BG	Bulgaria	HU	Hungary	ML	Mali	TT	Trinidad and Tobago
BJ	Benin	IE	Ireland	MN	Mongolia	UA	Ukraine
BR	Brazil	IL	Israel	MR	Mauritania	UG	Uganda
BY	Belarus	IS	Iceland	MW	Malawi	US	United States of America
CA	Canada	IT	Italy	MX	Mexico	UZ	Uzbekistan
CF	Central African Republic	JP	Japan	NE	Niger	VN	Viet Nam
CG	Congo	KE	Kenya	NL	Netherlands	YU	Yugoslavia
CH	Switzerland	KG	Kyrgyzstan	NO	Norway	ZW	Zimbabwe
CI	Côte d'Ivoire	KP	Democratic People's Republic of Korea	NZ	New Zealand		
CM	Cameroon			PL	Poland		
CN	China	KR	Republic of Korea	PT	Portugal		
CU	Cuba	KZ	Kazakstan	RO	Romania		
CZ	Czech Republic	LC	Saint Lucia	RU	Russian Federation		
DE	Germany	LI	Liechtenstein	SD	Sudan		
DK	Denmark	LK	Sri Lanka	SE	Sweden		
EE	Estonia	LR	Liberia	SG	Singapore		

A PROCESS FOR PREPARING HUMAN PROINSULIN

BACKGROUND OF THE INVENTION

Field of the Invention

The present invention relates to a process for preparing human proinsulin, more specifically, to a process for preparing human proinsulin which is represented as the following chemical formula(I):



(I)

wherein,

R is an amino acid residue or a peptide which is degradable enzymatically or chemically; and,

X is a linkage of an amino group of A-1 in insulin A chain and a carboxyl group of B-30 in insulin B chain which can be separated from the A chain or the B chain enzymatically or chemically, provided that a region from A-1

to A-21 is the insulin A chain and a region from B-1 to B-30 is the insulin B chain.

In the chemical formula(I), there exist 3 disulfide bonds(between A-6 and A-11, between A-7 and B-7, and between A-20 and B-19) formed from 6 cysteine residues which are present in the A chain and the B chain.

Description of the Prior Art

In general, human insulin precursor("proinsulin") has been prepared in the course of manufacturing mature insulin("insulin") by the recombinant DNA technology which comprises a step of inserting a structural gene into a plasmid DNA of E. coli.

As shown in Figure 1, a fusion protein containing the proinsulin is expressed in the form of inclusion body in E. coli, and the inclusion bodies obtained by centrifugation after lysis of the cells are washed with non-ioninc or ionic detergent, or with a denaturant at a low concentration. Such a treatment accompanied by centrifugation is repeated to result in increase of purity of the desired protein(see: Mukhopadhyay, A. et al., Advances in Biochemical Engineering/Biotechnology, 56, 61-108, 1997). In order to minimize intermolecular hydrophobic interaction and formation of incorrect disulfide bonds, the washed inclusion bodies are dissolved in a denaturant such as urea or guanidine·HCl solution containing a reducing agent such as dithiothreitol(DTT) or 2-mercaptoethanol, or in NaOH(see: Fischer et al., Biotechnology & Bioengineering, 41, 3-13, 1993). The dissolved inclusion bodies are centrifuged at a high speed, and the supernatant is diluted with cold water to recover the inclusion bodies as a precipitate(see: EP 0 055 945 A2). The inclusion bodies thus obtained contain a fusion protein of proinsulin and a heteroprotein such as β-galactosidase, which are linked by a cross-linkage of methionine residue. The fusion protein is treated with cyanogen bromide(CNBr), and substitution

of six(6) -SH groups present in proinsulin with $-SSO_3^-$ groups follows to give proinsulin S-sulfonate. Such a sulfonation step leads to increase in stability of insulin precursor and efficiency of a later refolding reaction(see: EP 0 055 945 A2). The proinsulin S-sulfonate is refolded to have a native conformation by using reducing agents such as 2-mercaptoethanol, DTT, etc., or a redox system such as glutathione(see: Fischer et al., Biotechnology & Bioengineering, 41, 3-13, 1993). The native proinsulin thus obtained is converted into biologically active insulin by removing X(or C chain) which links A chain and B chain through the treatment of trypsin and carboxypeptidase B(see: Kemmler W., et al., J.B.C., 246, 6786-6790, 1971). Finally, insulin is purified through a reverse-phase high performance liquid chromatography(RP-HPLC), etc.(see: Kroeff, E.P., et al., J. Chromatogr., 461, 45-61, 1989) and crystallized by the technique of Zn-crystallization(see: Mirsky, et al., J. Clinical Investigation, 42, 1869-1872, 1963; Brader M.L., TIBS, 16, 341-345, 1991).

The conventional process for preparing proinsulin or insulin is, however, proven to be less satisfactory in the senses that: it accompanies complicated steps of dissolution and sulfonation, purification, concentration and desalting; and, it employs an inefficient refolding reaction, which results in decreased yield of the desired protein. Accordingly, there are strong reasons for exploring and developing an improved process for preparing proinsulin or insulin in a simple and efficient manner, while preserving its biological activity.

SUMMARY OF THE INVENTION

The present inventors have made an effort to solve the problems of the conventional processes for preparing human recombinant proinsulin expressed in the form of inclusion body, and successfully established a process for preparing human pronisulin whose steps of dissolution and sulfonation, purification, concentration and desalting are remarkably

simplified, while increasing the efficiency of refolding reaction.

The primary object of the present invention is, therefore, to provide a simple process for preparing human recombinant proinsulin with a good reproducibility.

BRIEF DESCRIPTION OF DRAWINGS

The above and the other objects and features of the present invention will become apparent from the following descriptions given in conjunction with the accompanying drawings, in which:

Figure 1 is a schematic diagram showing a conventional process for preparing human insulin from a fused human insulin precursor which is expressed in recombinant E. coli.

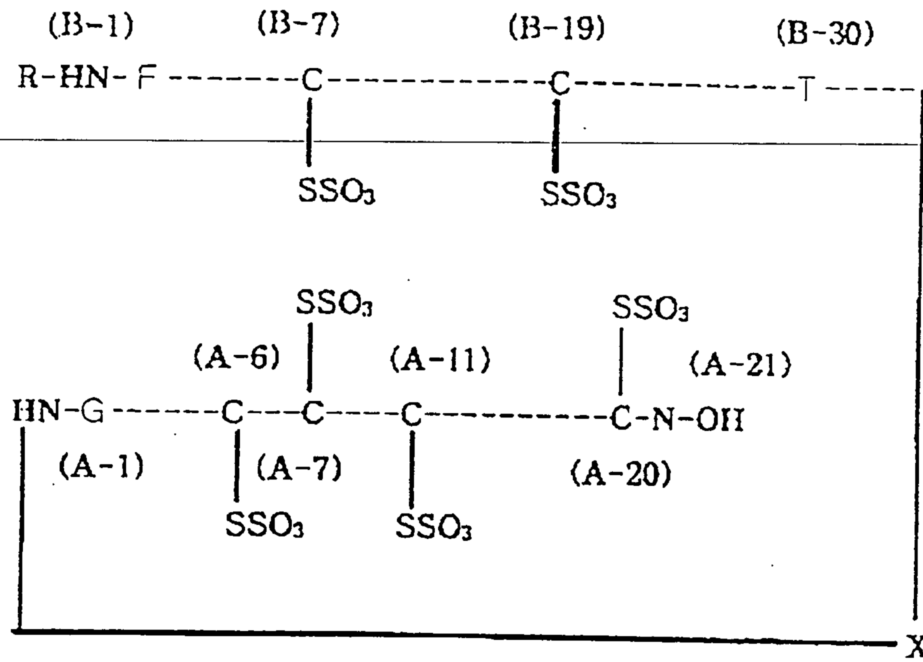
Figure 2 is a schematic diagram showing a process for preparing human insulin from a fused human insulin precursor which is expressed in recombinant E. coli, in accordance with the present invention.

Figure 3 depicts a refolding system employed in the process for preparing human proinsulin of the invention.

DETAILED DESCRIPTIION OF THE INVENTION

In accordance with the process for preparing human proinsulin of the present invention, human insulin precursor expressed in the form of inclusion body, is treated with sodium tetrathionate($Na_2S_4O_4$) and sodium sulfite(Na_2SO_3) during a step of dissolution of the inclusion body in urea or guanidine·HCl solution, which results in substitution of -SH groups in cysteine

residues of the insulin precursor with $-SSO_3^-$ groups, to give proinsulin S-sulfonate represented as the following chemical formula(II), which is converted into proinsulin represented as the chemical formula(I) by reacting the proinsulin S-sulfonate with 2-mercaptoethanol in an aqueous medium.



(II)

wherein,

R and X are the same as described as above.

The process for preparing human proinsulin of the invention is described in more detail accompanied with Figures 2 and 3. In the process for preparing human proinsulin, all steps are preferably carried out at a low temperature of about 4°C, though they may be performed at a room temperature for the practitioner's convenience.

<u>Step 1</u>: Purification of inclusion bodies

In order to prepare a recombinant human insulin, the present inventors used a fusion protein of a modified β-galactosidase

and proinsulin which is expressed in E. coli (see: Korean patent publication No. 94-1855). E. coli cells which express the fusion protein in the form of inclusion body are suspended in a buffer solution for lysis in a ratio of 1:5 to 10(w/v), and lysed under a pressure of about 9,000psi. The inclusion bodies are centrifuged and washed by using Triton X-100 and distilled water, and centrifuged to obtain purified inclusion bodies.

Step 2: Dissolution and sulfonation

The purified inclusion bodies are dissolved in 0.02 to 0.1M Tris buffer solution (pH 8 to 10) containing a denaturant of 6 to 8M urea or guanidine·HCl in a ratio of 1:10 to 20(w/v), more preferably 1:5 to 10(w/v), while adding 0.1 to 0.6M, more preferably 0.2 to 0.5M sodium sulfite (Na_2SO_3) and 0.01 to 0.1M, more preferably 0.05 to 0.1M sodium tetrathionate ($Na_2S_4O_4$). Then, the mixed solution containing the inclusion bodies are stirred to induce sulfonation of insulin precursor, which results in substitution of -SH groups of the insulin precursor with $-SSO_3^-$ groups. In this step, pH and temperature are maintained in the ranges of pH 7.0-9.5 and 4-8°C, respectively. Finally, sulfonated proinsulin fusion protein is obtained by substituting -SH groups in cysteine residues of the proinsulin with $-SSO_3^-$ groups.

Step 3: Treatment with cyanogen bromide

After the sulfonation reaction, the reaction mixture is centrifuged at 12,000 rpm for 30 minutes to remove precipitates. Cold water is added to the supernatant in a ratio of 5 to 20:1 (v/v), and pH is adjusted to 5 to 6 to give a precipitate. The precipitated protein is dissolved in 70% (v/v) formic acid to reach a concentration of 10-30mg/ml, and is subsequently treated with cyanogen bromide so that the molar ratio of the cyanogen bromide to protein is 100:1. This results in separation of the proinsulin S-sulfonate from the fusion protein. And then,

drying is carried out under a reduced pressure.

Step 4: Ion-exchange chromatography

Proinsulin S-sulfonate is dissolved in 20mM Tris buffer (pH 8.0) containing 1mM EDTA and 7M urea to reach a concentration of 30mg/ml and loaded onto a DEAE-Sephacel resin equilibrated with the same buffer. Then, elution is made by employing a concentration gradient of 0-1M NaCl, to give the proinsulin S-sulfonate in the concentration range of 0.3-0.5M NaCl.

Step 5: Refolding (Conversion of proinsulin S-sulfonate to proinsulin)

The purified proinsulin S-sulfonate is diluted with 50mM glycine buffer (pH 10.6) containing 1M urea at a concentration of 1-10mg/ml, without desalting or pretreatment. Then, nitrogen gas is purged to remove oxygen and the chamber is sealed well. In another chamber, 2-mercaptoethanol is added to 50mM glycine buffer (pH 10.6) containing 1M urea in an equivalent ratio of 1 to 3 against $-SSO_3^-$ groups of proinsulin S-sulfonate. And then, the protein solution and the buffer solution containing 2-mercaptoethanol are mixed rapidly in a ratio of 1:1 (v/v) by connecting the two chambers to a mixing cell having a volume of 0.1ml to 10L, and the refolding reaction mixture is introduced into a reservoir while stirring slowly and reacted for 15 to 20 hours at 4-5°C (see: Figure 3). By carrying out the refolding step, at least 80% of proinsulin S-sulfonate can be converted into native proinsulin.

Step 6: Adsorption chromatography

To purify and concentrate the refolded proinsulin, the refolding reaction mixture containing the refolded proinsulin is contacted with a polar methacrylate resin of HP-2MG, while adjusting the reaction mixture in a pH range of 3 to 4, so that

8g of the mixed proteins containing the refolded proinsulin can contact with 1 liter of the resin. In this connection, protein concentration of the loading sample is controlled in a range of 0.1 to 5mg/ml, depending on the condition of the proinsulin refolding reaction. After adsorption, the resin is washed with acetic acid buffer(pH 3 to 4), and the refolded proinsulin is eluted using an aqueous acetic acid buffer(pH 3 to 4) containing 15 to 50%(v/v), preferably 30 to 50%(v/v) of acetone. Then, the refolded human proinsulin is recovered in a powder form from the eluent for later use by the evaporation and freeze-drying of pooled active fractions, or is recovered in a precipitate form by the addition of zinc chloride to the pooled active fractions in a final concentration of 0.004 to 4%(w/v), preferably 0.004 to 0.04%(w/v) after adjusting pH of the eluent to 5 to 7, preferably 5 to 6 with NaOH, and centrifugation.

The process for preparing a human insulin precursor of the invention has following advantages over the conventional processes: first, dissolution and sulfonation are carried out simultaneously, which results in simplification of steps for mass-production; secondly, denaturation such as gellation occurring by intermolecular polymerization in a sample of high concentration can be successfully prevented; thirdly, the problem of decrease in solubility of a sample caused by incorrect intermolecular disulfide bonds during dissolution can be solved fundamentally. Although this problem can be overcome by using reducing agents such as 2-mercaptoethanol or DTT, sulfonation by sulfite and tetrathionate is more stable and gives better solubility than the treatment with reducing agents; fourthly, -SH groups of cysteine residues are substituted with $-SO_3^-$ groups prior to cyanogen bromide treatment, which prevents irreversible denaturation of cysteine residues which may occur in dissolution step and the following steps, e.g., denaturation of cysteine to cysteic acid(<u>see</u>: USP 4,451,396); fifthly, cyanogen bromide treatment and evaporation are followed by ion-exchange chromatography or adsorption chromatography

without any pretreatment such as desalting, and it is therefore advantageous in a continuous industrial scale process.

The present invention is further characterized by the efficient refolding reaction which is one of the most crucial steps for industrial scale manufacture of insulin. Particularly, increase in yield and good reproducibility during scale-up are obtained by the continuous reaction employing a mixing cell. Also, native proinsulin can be purified, concentrated and desalted simultaneously from a reaction mixture by using an adsorption resin for industrial use after the refolding reaction.

In the conventional processes for preparing human insulin precursor which essentially include the refolding reaction, cysteine residues of the polypeptide represented as the chemical formula(I) described above are sulfonated to give proinsulin S-sulfonate, which is reacted with 1 to 5 equivalents of 2-mercaptoethanol per $-SSO_3^-$ group under a neutral or alkaline condition of pH 7 to 11.5(see: USP 4,430,266). Such a batch-type reaction results in a refolding yield of about 60%, though it can be increased to a level of 80% through a recycling system, by employing the complicated steps of adjusting pH condition to 4 to 6 after refolding reaction to obtain intermediate substances showing incorrect refolding in a form of aggregate, sulfonating again and refolding(see: USP 4,801,684). However, in accordance with the present invention, up to 80% of the proinsulin S-sulfonate can be converted into native proinsulin through only one step of refolding reaction, which results in remarkable simplification of the step and increase in yield.

Many studies on the refolding reaction have revealed that: a principal cause of decreasing the refolding yield of proteins is the formation of aggregates by non-covalent bonds between exposed hydrophobic residues of intermediate substances during refolding reaction; and, the formation of aggregates is largely influenced by several factors such as protein concentration, reaction volume, temperature, pH, etc., while the protein concentration affects the productivity largely. Thus, if the

refolding reaction of protein is carried out at a low concentration to obtain a high yield, industrial scale manufacture cannot be realized. Therefore, a variety of alternative methods for refolding a recombinant protein have been proposed in the art. For example, a method for adding aggregation inhibitor such as arginine, detergent such as polyethyleneglycol or denaturant, and a method for stepwise increase in protein concentration during the refolding reaction have been suggested successively.

The said methods, however, have revealed a shortcoming that homogeneous mixing of reactants such as proinsulin S-sulfonate and 2-mercaptoethanol is not performed rapidly, which can lead to the aggregation to decrease refolding yield, since a large reaction volume is required for the refolding reaction of insulin on industrial scale. Under the circumstances, in order to reduce the time required for equilibrium during mixing of proinsulin S-sulfonate and 2-mercaptoethanol, the present inventors have carried out a refolding reaction by mixing them continuously in a mixing cell of a small volume, which finally provides proinsulin in a high yield, even though a highly concentrated proinsulin S-sulfonate is employed.

In order to purify the refolded proinsulin after the refolding reaction, gel filtration chromatography such as Sephadex G-50 and ion-exchange chromatography are generally employed, which essentially require a step of changing of buffer solution and a step of desalting to remove remaining salts(see: USP 4,430,266). The conventional desalting methods include gel filtration, dialysis, ultrafiltration, etc. Among them, gel filtration employs polydextran gel such as Sephadex G-25 to separate substances depending on molecular weight or structure of the substances, based on the difference in retention time for the substances to pass through the gel. On the other hand, a dialysis membrane, instead of the gel, is used for dialysis technique, and a cartridge such as hollow fiber and cassette, and a disc membrane are used for ultrafiltration.

However, the said methods have revealed the following

several shortcomings: That is, in carrying out the gel filtration, sample capacity depends on gel volume packed in a column rather than quantity or concentration of the sample(capacity: 10 to 25% of gel volume). Thus, if a dilute sample is employed in the gel filtration, the column size becomes larger. Also, the eluted sample is diluted, which gives troubles in the later steps. On the other hand, the dialysis technique has drawbacks of sample loss caused by nonspecific binding of the sample to the dialysis membrane and the limited capacity as well. Ultrafiltraion has also disadvantages of requirement of a specific equipment, sample loss caused by nonspecific binding, fouling and plugging, and decrease in flow rate, although it has advantages of high capacity and efficient concentration capability.

In accordance with the present invention, a step of desalting, unlike the conventional processes, is performed employing adsorption chromatography, which successfully solves the said problems, i.e., limited capacity, dilution of the sample, nonselective binding, etc. Practically, the refolding reaction solution containing active proinsulin is adjusted to an acidic condition of pH 3 to 4, and loaded onto a polar methacrylate resin to recover almost all of the refolded proinsulin by using a buffer solution(pH 3 to 4) containing 15 to 50%(v/v) acetone. In this connection, the polar methacrylate resin under the trademark of HP-2MG which is commercially available from Mitsubishi Chemical Co. is preferably employed for the adsorption of organic substances showing relatively high polarity. The adsorption/elution step is carried out in a column for the best efficiency of desalting, concentration and purification. However, it may be performed in a way of batch or column. In this step, more than 90% of yield, and efficient concentration of several to dozens times can be accomplished, depending on the concentration of the loading sample.

In short, the refolded proinsulin can be economically desalted, concentrated, and purified in one step, and the eluent itself can be directly used in later steps.

The present invention is further illustrated in the following examples, which should not be taken to limit the scope of the invention.

Example 1: Purification of inclusion bodies

E. coli cells which express proinsulin fusion proteins in a form of inclusion body(see: Korean patent publication No. 94-1855) were suspended in 0.1M Tris buffer(pH 7.9) containing 50mM EDTA, 10% sucrose and 0.1mM PMSF in a ratio of 1:5 to 10(w/v) and lysed under a pressure of 9,000psi. The lysate was centrifuged at 5,000 rpm for 30 minutes at 4°C to obtain precipitate. 300g(wet weight) of the precipitate containing inclusion bodies was washed with 10 volume of 2% Triton-X100 and distilled water, and centrifuged to obtain purified inclusion bodies.

Example 2: Dissolution of inclusion bodies by alkali

The inclusion bodies obtained in Example 1 were suspended uniformly in 20 volumes of distilled water, stirred for 3 hours, and centrifuged at 12,000 rpm for 30 minutes to remove precipitate. pH of the supernatant thus obtained was adjusted to 5.5 with 1M HCl and centrifuged at 5,000 rpm for 30 minutes to obtain precipitate. The precipitated protein was dissolved in 70%(v/v) formic acid to reach a concentration of 10mg/ml. Then, cyanogen bromide was added in a molar ratio of 100:1 with respect to the amount of the protein, and stirred for 12 hours at 25°C. And then, evaporation under a reduced pressure was carried out for complete drying and the protein thus obtained was dissolved in 20mM Tris buffer(pH 9.5) containing 7M urea. Sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively, and stirred for 6 hours. And then, HPLC analysis was carried out to determine concentration of the sulfonated proinsulin(see: Table 1).

Example 3: Dissolution of inclusion bodies by guanidine·HCl and reducing agents

The inclusion bodies obtained in Example 1 were suspended in 10 volume of several buffer solutions containing a denaturant as followings: first, they were dissolved in 20mM Tris buffer(pH 9.5) containing 6-7M guanidine·HCl and 1mM EDTA; secondly, they were dissolved in 20mM Tris buffer(pH 9.5) containing 6-7M guanidine·HCl and 1mM EDTA, and 1mM 2-mercaptoethanol was added; thirdly, they were dissolved in 20mM Tris buffer(pH 9.5) containing 6-7M guanidine·HCl and 1mM EDTA, and sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively. And then, each solution was stirred for 12 hours at 4°C, and centrifuged at 12,000 rpm for 30 minutes to remove precipitate. Then, about 10 volume of cold water was added to the supernatant thus obtained and centrifuged at 5,000 rpm for 30 minutes to obtain precipitate. The precipitated protein was dissolved in 70%(v/v) formic acid to reach a concentration of 10mg/ml. Then, cyanogen bromide was added in a molar ratio of 100:1 with respect to the amount of the protein, and stirred for 12 hours at 25°C. And then, evaporation under a reduced pressure was carried out for complete drying and the protein thus obtained was dissolved in 20mM Tris buffer(pH 9.5) containing 7M urea. Sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively, and stirred for 6 hours at 25°C. And then, HPLC analysis was carried out to determine the concentration of the sulfonated proinsulin(see: Table 1).

Example 4: Dissolution of inclusion bodies by urea and reducing agents

The inclusion bodies obtained in Example 1 were suspended in 10 volume of several buffer solutions containing a denaturant as followings: first, they were dissolved in 20mM Tris buffer(pH 9.5) containing 7-8M urea and 1mM EDTA; secondly, they were

dissolved in 20mM Tris buffer(pH 9.5) containing 7-8M urea and 1mM EDTA, and 1mM 2-mercaptoethanol was added; thirdly, they were dissolved in 20mM Tris buffer(pH 9.5) containing 7-8M urea and 1mM EDTA, and sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively. And then, each solution was stirred for 12 hours at 4°C, and centrifuged at 12,000 rpm for 30 minutes to remove precipitate. Then, about 10 volume of cold water was added to the supernatant thus obtained and centrifuged at 5,000 rpm for 30 minutes to obtain precipitate. The precipitated protein was dissolved in 70%(v/v) formic acid to reach a concentration of 10mg/ml. Then, cyanogen bromide was added in a molar ratio of 100:1 with respect to the amount of the protein, and stirred for 12 hours at 25°C. And then, evaporation under a reduced pressure was carried out for complete drying and the protein thus obtained was dissolved in 20mM Tris buffer(pH 9.5) containing 7M urea. Sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively, and stirred for 6 hours at 25°C. And then, HPLC analysis was carried out to determine the concentration of the sulfonated proinsulin(see: Table 1).

Table 1: Effect of various dissolving methods in Examples 2 to 4 on sulfonation*

Treatment	Amount of protein after dissolution (g)	Yield of sulfonation (%) (sulfonated proinsulin g/amount of protein)
Control	10.2	28.6
Dissolution by alkali	7	6.7
Dissolution by guanidine·HCl	-	-
Dissolution by guanidine·HCl + 1mM 2-mercaptoethanol	7.1	24
Dissolution by guanidine·HCl + sulfite + tetrathionate	6.3	48
Dissolution by urea	7.0	31.5
Dissolution by urea + 1mM 2-mercaptoethanol	7.1	28
Dissolution by urea + sulfite + tetrathionate	6.2	45.2

*: Each sample employed 30g(in wet weight) of inclusion bodies washed with Triton X-100 and cold distilled water, and the same amount of inclusion bodies treated with CNBr only, is employed as control.

As can be seen in Table 1, addition of sodium sulfite and sodium tetrathionate after dissolution by urea or guanidine·HCl resulted in an increased yield of sulfonated proinsulin which is 1.5 to 2 times higher than the control. On the other hand, when dissolution was carried out by only guanidine·HCl without any reducing agent, gellation occurred in the course of adding 70%(v/v) formic acid. Therefore, the amount of protein after dissolution and yield of sulfonation could not be determined. Such a result may be caused by intermolecular hydrophobic interaction or polymerization by formation of disulfide bond. Also, addition of 2-mercaptoethanol resulted in a considerable decrease in yield, which may also be caused by the same reasons described above.

It was clearly demonstrated that: the intermolecular interaction can be prevented through the substitution with $-SSO_3^-$ groups in sulfonated proinsulin fusion protein to give negative charge to the whole molecule; and, dissolution by alkali influences stability of the proteins. On the other hand, addition of sulfite and tetrathionate after dissolution by urea or guanidine·HCl, has no remarkable difference in the yield of sulfonated proinsulin. Therefore, use of urea for dissolution on industrial scale would result in a remarkable reduction in cost.

In addition, HPLC analysis of the sample which was obtained through the steps of dissolution and sulfonation, cyanogen bromide treatment and dissolution in 20mM Tris buffer containing 7M urea, has revealed that: the addition of sulfite and tetrathionate after cyanogen bromide treatment did not increase the yield of sulfonation.

<u>Example 5</u>: Dissolution of inclusion bodies by urea and sulfonation

Based on the results in Example 2 to 4 described aboves, the inclusion bodies were dissolved in a urea solution and sulfonation was carried out. That is, 110g(in wet weight) of the purified inclusion bodies were dissolved in 10 volume of 20mM Tris buffer(pH 8.5) containing 8M urea and 1mM EDTA. Then, sodium sulfite and sodium tetrathionate were added at a final concentration of 0.3M and 0.1M, respectively, stirred for 12 hours at 4°C, and centrifuged at 12,000 rpm for 30 minutes to remove precipitate. And then, about 10 volume of cold water was added to the supernatant thus obtained, and pH of the solution was adjusted to about 5.5 with 2N HCl solution, and centrifuged at 5,000 rpm for 30 minutes to give precipitate of 250g in wet weight. Quantitative assay of protein revealed that about 40g of protein was finally obtained.

Example 6: Treatment with cyanogen bromide

The precipitated protein was dissolved in 2L of 70%(v/v) formic acid. Then, cyanogen bromide(CNBr) was added in a molar ratio of 100:1 with respect to the amount of the protein, and stirred for 12 hours at 25°C. And then, evaporation under a reduced pressure was carried out for complete drying. The protein thus obtained was dissolved in 20mM Tris buffer(pH 8.0) containing 7M urea, and analyzed by HPLC.

Example 7: Anion-exchange chromatography

DEAE-Sephacel was packed in a column(2.5 x 30cm) at a flow rate of 1.5 column volume per hour, and equilibrated with 20mM Tris buffer(pH 8.0) containing 7M urea. Then, the sample obtained in Example 6 was loaded onto the column at a ratio of 20mg per 1ml of the resin, and the column was washed with 1 column volume of the equilibrium buffer. The protein was eluted by a concentration gradient by using the equilibrium buffer containing 0-1M NaCl. Then, the eluents collected at 0.35-0.45M NaCl were analyzed by HPLC, which revealed that purity was 80% or more and recovery rate was 91%.

Example 8: Refolding of proinsulin S-sulfonate

1g of proinsulin S-sulfonate which was obtained by sulfonating an active recombinant proinsulin purified by RP-HPLC, was dissolved in 500ml of 50mM glycine buffer(pH 10.6). Then, nitrogen gas was purged to remove oxygen and the chamber was sealed well. In another chamber, 104μl of 2-mercaptoethanol was added to 500ml of 50mM glycine buffer(pH 10.6), nitrogen gas was also purged to remove oxygen and the chamber was sealed well. And then, the two solutions were rapidly introduced into a mixing cell having a volume of 1ml at a flow rate of 50ml/hr while stirring. The refolding reaction solution thus mixed was introduced at a flow rate of 100ml/hr into a reservoir purged

with N_2 gas, stirred slowly, and reacted for 18 hours at 4°C. After the reaction was completed, the solution was acidified to give pH 2.9±0.1 by using 2M HCl. HPLC analysis revealed that refolding yield was 55%.

Example 9: Effect of protein concentration on refolding

Effect of protein concentration on the yield of refolding(i.e., conversion of proinsulin S-sulfonate to proinsulin) was investigated, through a series of reactions performed analogously as in Example 8 except for the protein concentration(see: Table 2).

Table 2: Effect of protein concentration on the yield of refolding

Protein concentration (mg/ml)	Yield (%)	Protein concentration (mg/ml)	Yield (%)
0.1	95	1	53
0.2	90	2	20
0.5	83	4	8

Example 10: Effect of $-SH:-SSO_3^-$ ratio on refolding

Effect of $-SH:-SSO_3^-$ ratio on the yield of refolding was investigated through a series of reactions performed analogously as in Example 8 except for the $-SH:-SSO_3^-$ ratio(see: Table 3).

Table 3: Effect of $-SH:-SSO_3^-$ ratio on the yield of refolding

$-SH:-SSO_3^-$ ratio	Yield (%)	$-SH:-SSO_3^-$ ratio	Yield (%)
1	41	2	54
1.5	59	3	30

Example 11: Effect of urea concentration on refolding

Effect of urea concentration on the yield of refolding was investigated through a series of reactions performed analogously as in Example 8 except for urea concentrations(see: Table 4).

Table 4: Effect of urea concentration on the yield of refolding

Urea concentration (M)	Yield (%)	Urea concentration (M)	Yield (%)
0	52	0.5	79
0.25	67	1.0	83

Example 12: Refolding of proinsulin S-sulfonate purified by ion-exchange chromatography

Eluent containing 10g of proinsulin S-sulfonate obtained in Example 7 was diluted with 50mM glycine buffer(pH 10.6) containing 1M urea to reach a final volume of 5L. Then, nitrogen gas was purged to remove oxygen and the chamber was sealed well. In another chamber, 781μl of 2-mercaptoethanol was added to 5L of 50mM glycine buffer containing 1M urea, nitrogen gas was purged to remove oxygen and the chamber was sealed well. And then, the two solutions were mixed rapidly, by introducing into a mixing cell having a volume of 1ml at a flow rate of 500ml/hr while stirring. The refolding reaction mixture was introduced at a flow rate of 1L/hr into a reservoir deaerated with N_2 gas, stirred slowly, and reacted for 18 hours at 4°C. After the reaction was completed, the solution was acidified to give pH 2.9±0.1 by using 2M HCl. HPLC analysis revealed that refolding yield was 81%.

Example 13: Purification of human recombinant proinsulin by adsorption chromatography

HP-2MG resin(Mitsubishi Chemical Co., Japan), a polar methacrylate resin, was swollen in a ratio of 1g of a resin per 5ml of acetone for 6 hours at room temperature. Then, the resin was sufficiently washed with 0.1N NaOH, distilled water, 0.1N HCl, distilled water and 20mM acetic acid(pH 3.2±0.2) in order, and packed in a column. And then, the column was equilibrated with 3 column volume of an equilibrium buffer(20mM acetic acid, pH 3.2±0.2) at a flow rate of 1 column volume per hour. Then, the reaction solution containing refolded proinsulin obtained in Example 12 was loaded onto the column in a ratio of 8g of the protein per 1L of the resin, and the column was washed with 1 column volume of 20mM acetic acid buffer(pH 3.2±0.2). And then, the refolded proinsulin was eluted with the same buffer containing 30% acetone. As a result, 92% or more of the refolded proinsulin was recovered, while being free from impurities such as glycine and urea. Also, HPLC and quantitative protein assay revealed that protein was concentrated in about 10-fold with an increased purity of about 1.3-fold. Then, the said eluent containing active proinsulin was evaporated to remove acetone and freeze-dried, or pH of the eluent was adjusted to 5.4 with 1N NaOH and zinc chloride was added at a final concentration of 0.04%(w/v) to recover the refolded proinsulin.

As clearly illustrated and demonstrated as above, the present invention provides a process for preparing human proinsulin whose steps of dissolution and sulfonation, concentration, desalting and purification are remarkably simplified, while increasing the yield of refolding reaction. In accordance with the present invention, human recombinant insulin precursor can be manufactured with a good reproducibility.

WHAT IS CLAIMED IS:

1. A process for preparing human proinsulin which comprises the steps of:

(i) suspending in a buffer solution *E. coli* cells which express proinsulin fusion proteins in the form of an inclusion body and lysing the said cells to obtain the inclusion body;

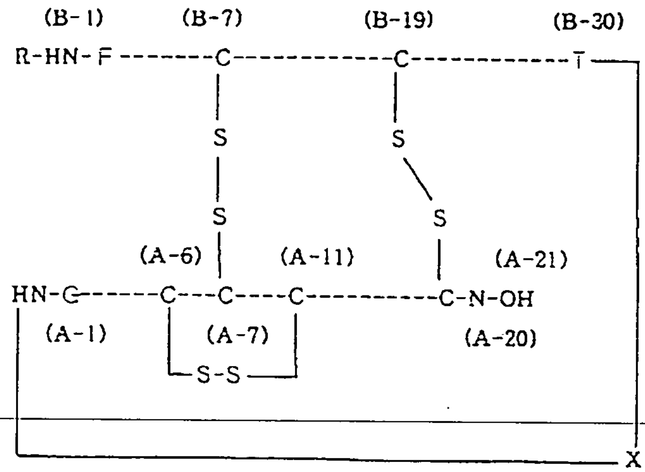
(ii) suspending the said inclusion body obtained in step(i) in a buffer solution containing a denaturant, while sulfonating the proinsulin fusion proteins with sodium sulfate and sodium tetrathionate, to obtain a fusion protein of proinsulin S-sulfonate represented as the following formula(II);

(iii) centrifuging the fusion protein of proinsulin S-sulfonate obtained in step(ii) to produce a precipitate, dissolving the precipitate in formic acid, then cleaving proinsulin S-sulfonate from the said fusion protein by treating with cyanogen bromide and drying under a reduced pressure;

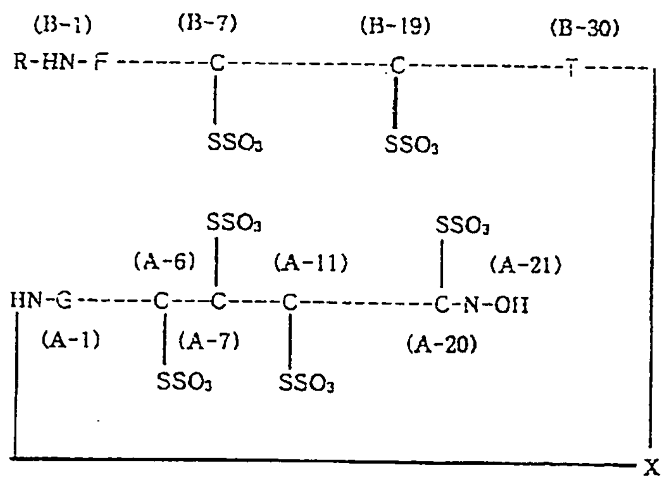
(iv) dissolving the dried proinsulin S-sulfonate obtained in step(iii) in a buffer and purifying the said proinsulin S-sulfonate on anion-exchange chromatography;

(v) diluting the said purified proinsulin S-sulfonate obtained in step(iv) in a first buffer solution, purging nitrogen gas to remove oxygen to obtain a first mixture, mixing the first mixture with a second buffer solution containing 2-mercaptoethanol in a mixing cell, and stirring in a reservoir to obtain a refolding reaction mixture containing proinsulin represented as the following formula(I); and,

(vi) applying the refolding reaction mixture obtained in step(v) to an adsorption chromatography resin and eluting by an aqueous solution, to give refolded human proinsulin,



(I)



(II)

wherein,

R is an amino acid residue or a peptide which is degradable enzymatically or chemically; and,

X is a linkage of an amino group of A-1 in insulin A chain and a carboxyl group of B-30 in insulin B chain which can be separated from the A chain or the B chain enzymatically or chemically, provided that a region from A-1 to A-21 is the insulin A chain and a region from B-1 to B-30 is the insulin B chain.

2. The process for preparing human proinsulin of Claim 1, wherein the denaturant is urea or guanidine·HCl.

3. The process for preparing human proinsulin of Claim 1, wherein the concentration of the denaturant ranges from 6 to 8M.

4. The process for preparing human proinsulin of Claim 1, wherein the inclusion body is suspended in 0.02 to 0.1M Tris buffer solution(pH 8 to 10) containing the denaturant.

5. The process for preparing human proinsulin of Claim 1, wherein the concentrations of sodium sulfite and sodium tetrathionate range from 0.1 to 0.6M and from 0.01 to 0.1M, respectively.

6. The process for preparing human proinsulin of Claim 1, wherein the inclusion body is suspended in the buffer solution containing the denaturant in a ratio of 10 to 20(w/v).

7. The process for preparing human proinsulin of Claim 1, wherein the inclusion body is suspended in the buffer solution containing the denaturant at a reaction temperature of 4 to 8°C.

8. The process for preparing human proinsulin of Claim 1, wherein the dried proinsulin S-sulfonate is dissolved in Tris buffer(pH 7 to 9) containing 1mM EDTA and 7M urea and purified on anion-exchange chromatography equilibrated with the same buffer.

9. The process for preparing human proinsulin of Claim 1, wherein the purified proinsulin S-sulfonate is diluted with a glycine buffer solution(pH 9 to 11) containing 1M urea in a concentration of 0.1 to 10mg/ml.

10. The process for preparing human proinsulin of Claim 1, wherein 2-mercaptoethanol is added to a buffer solution

containing 1M urea in an equivalent ratio of 1 to 3 with respect to the $-SSO_3^-$ groups of proinsulin S-sulfonate.

11. The process for preparing human proinsulin of Claim 1, wherein the buffer solution containing diluted proinsulin S-sulfonate and the buffer solution containing 2-mercaptoethanol are mixed in a ratio of 1:1(v/v).

12. The process for preparing human proinsulin of Claim 10 or 11, wherein the buffer solution employed is 50mM glycine buffer solution(pH 10.6).

13. The process for preparing human proinsulin of Claim 11, wherein the mixing is carried out in a mixing cell having a volume of 0.1ml to 10L.

14. The process for preparing human proinsulin of Claim 1, wherein the adsorption chromatography employs a polar methacrylate resin.

15. The process for preparing human proinsulin of Claim 14, wherein the refolding reaction mixture is adsorbed to the polar methacrylate resin at a pH value of 3 to 4.

16. The process for preparing human proinsulin of Claim 14, wherein the polar methacrylate resin is washed with acetic acid buffer(pH 3 to 4) before the elution of the refolded human proinsulin is made.

17. The process for preparing human proinsulin of Claim 1, wherein the aqueous solution is acetic acid buffer solution(pH 3 to 4) containing 15 to 50%(v/v) acetone.

18. The process for preparing human proinsulin of Claim 1, wherein the refolded human proinsulin eluted by an aqueous solution is recovered from the eluent containing the refolded

proinsulin by the addition of zinc chloride.

19. The process for preparing human proinsulin of Claim 18, wherein the zinc chloride is added into the eluent containing the refolded proinsulin to a final concentration of 0.004 to 4%(w/v) at a pH value of 5 to 7.

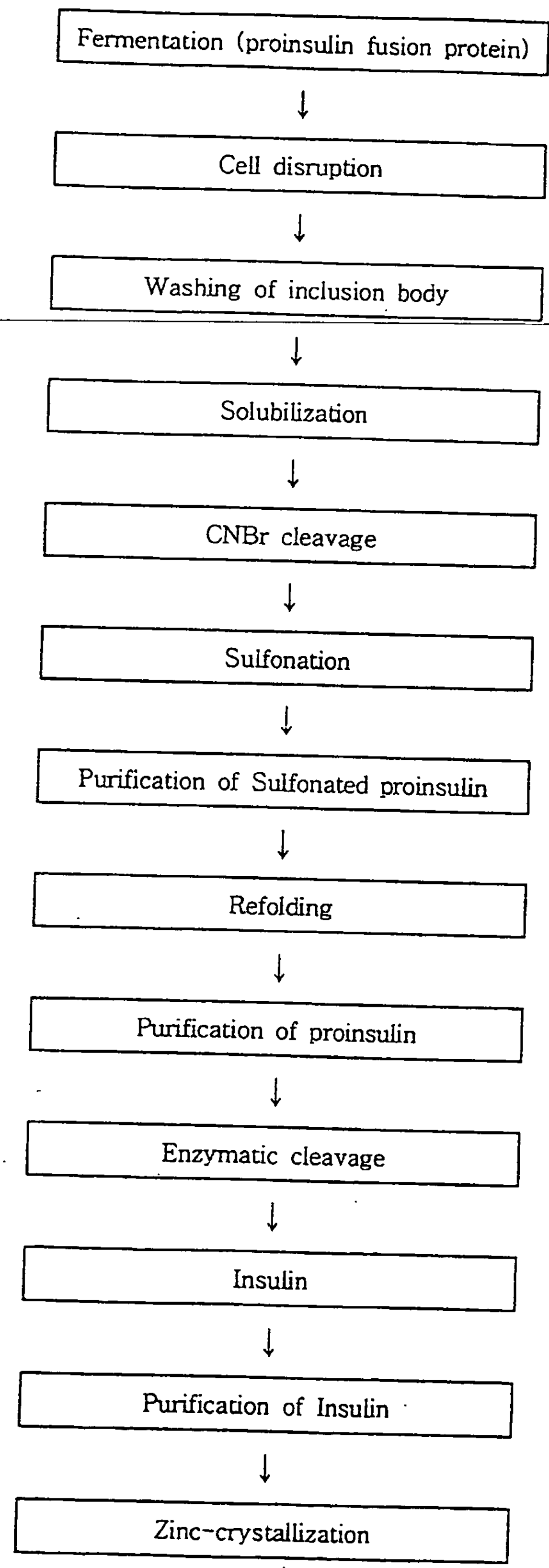

Fermentation (proinsulin fusion protein)
Cell disruption
Washing of inclusion body
Solubilization
CNBr cleavage
Sulfonation
Purification of Sulfonated proinsulin
Refolding
Purification of proinsulin
Enzymatic cleavage
Insulin
Purification of Insulin
Zinc-crystallization

Fig. 1

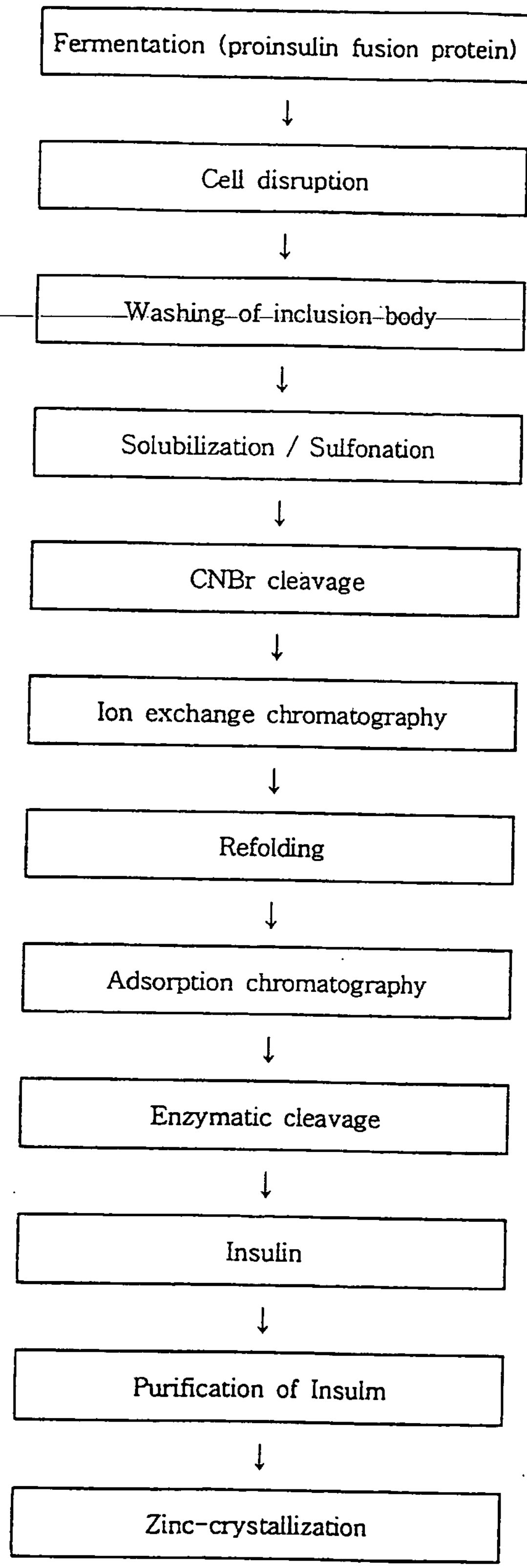

Fermentation (proinsulin fusion protein)
Cell disruption
Washing of inclusion body
Solubilization / Sulfonation
CNBr cleavage
Ion exchange chromatography
Refolding
Adsorption chromatography
Enzymatic cleavage
Insulin
Purification of Insulm
Zinc-crystallization

Fig. 2

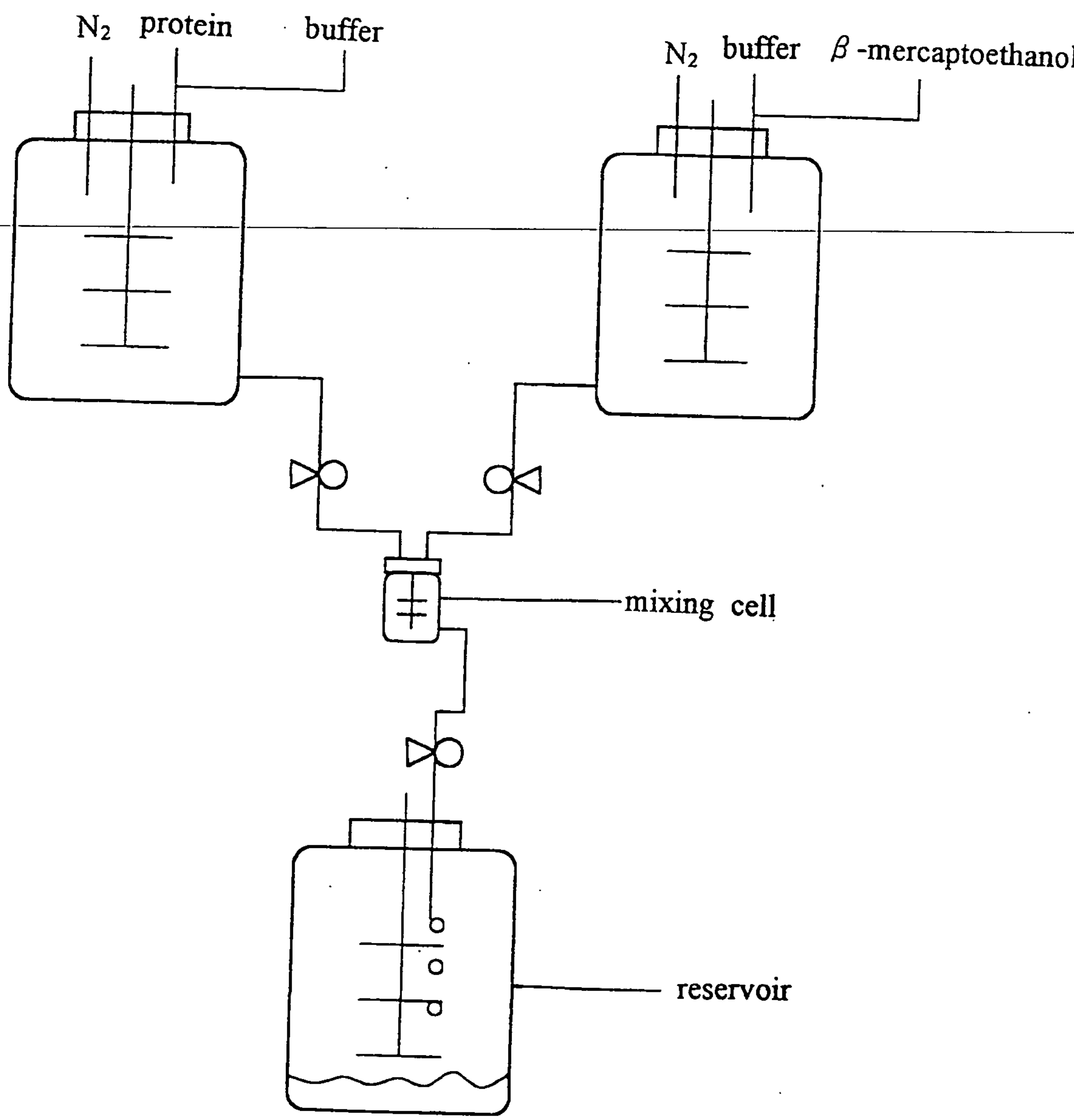

N2
protein
buffer
N2
buffer
β-mercaptoethanol
mixing cell
reservoir

Fig. 3

INTERNATIONAL SEARCH REPORT

International application No.
PCT/KR 98/00464

A. CLASSIFICATION OF SUBJECT MATTER

IPC^6: C 12 N 15/17, 1/06, 15/62

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

IPC^6: C 12 N 15/17, 1/06, 15/62

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

~~WPI; EPODOC~~

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
A	US 5 460 954 A (LEE et al.) 24 October 1995 (24.10.95), abstract.	1
A	EP 0 055 945 A2 (GENENTECH, INC.) 14 July 1982 (14.07.82), abstract.	1
A	US 4 430 266 A (FRANK) 07 February 1984 (07.02.84), abstract; claim 1.	1
A	US 5 426 036 A (KOLLER et al.) 20 June 1995 (20.06.95), abstract.	1

☐ Further documents are listed in the continuation of Box C. ☒ See patent family annex.

* Special categories of cited documents:
„A" document defining the general state of the art which is not considered to be of particular relevance
„E" earlier application or patent but published on or after the international filing date
„L" document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)
„O" document referring to an oral disclosure, use, exhibition or other means
„P" document published prior to the international filing date but later than the priority date claimed
„T" later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
„X" document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
„Y" document of particular relevance; the claimed invention cannot be considered to involve an inventive step when the document is combined with one or more other such documents, such combination being obvious to a person skilled in the art
„&" document member of the same patent family

Date of the actual completion of the international search	Date of mailing of the international search report
08 April 1999 (08.04.99)	15 April 1999 (15.04.99)

Name and mailing adress of the ISA/AT	Authorized officer
Austrian Patent Office Kohlmarkt 8-10; A-1014 Vienna Facsimile No. 1/53424/535	Wolf Telephone No. 1/53424/436

INTERNATIONAL SEARCH REPORT

Information on patent family members

International application No.
PCT/KR 98/00464

Im Recherchenbericht angeführtes Patentdokument Patent document cited in search report Document de brevet cité dans le rapport de recherche	Datum der Veröffentlichung Publication date Date de publication	Mitglied(er) der Patentfamilie Patent family member(s) Membre(s) de la famille de brevets	Datum der Veröffentlichung Publication date Date de publication
US A 5460954	24-10-95	KR B1 9509843	29-08-95
EP A2 55945	14-07-82	AU A1 79145/81	08-07-82
		DK A 5824/81	13-08-82
		EP A3 55945	15-09-82
		ES A1 508446	01-01-84
		ES A5 508446	25-01-84
		ES A1 8401777	16-03-84
		FI A 814172	03-07-82
		GR A 78327	26-09-84
		IL A0 64634	31-03-82
		IL A1 64634	31-03-86
		JP A2 57163352	07-10-82
		NZ A 199391	13-12-85
		PH A 20660	23-03-87
		ZA A 8108953	23-02-83
US A 4430266	07-02-84	AR A1 224933	29-01-82
		AT E 5400	15-12-83
		AU A1 68719/81	01-10-81
		AU B2 540644	29-11-84
		CA A1 1154435	27-09-83
		DD C 157612	24-11-82
		DE C0 3161475	29-12-83
		DK A 1364/81	28-09-81
		DK B 149863	13-10-86
		DK C 149863	01-06-87
		EG A 15310	31-12-85
		EP A1 37255	07-10-81
		EP B1 37255	23-11-83
		EP B2 37255	05-04-89
		ES A1 500747	16-01-82
		ES A5 500747	15-02-82
		ES A1 8201957	01-04-82
		FI A 810917	28-09-81
		GB A1 2073204	14-10-81
		GB B2 2073204	21-09-83
		GR A 73517	08-03-84
		HU B 185249	28-12-84
		IE B 51172	29-10-86
		IL A0 62486	20-05-81
		IL A1 62486	31-12-84
		JP A2 56150051	20-11-81
		JP B4 1048278	18-10-89
		KR B1 8400946	01-07-84
		NO A 811039	28-09-81
		NO B 151898	18-03-85
		NO C 151898	26-06-85
		NZ A 196609	03-02-84
		PH A 16156	18-07-83
		PL A1 230292	27-11-81
		PL B1 127843	30-11-83
		PT A 72732	01-04-81
		PT B 72732	23-03-82
		RO F 81951	21-02-84
		SU A3 1301319	30-03-87
		YU A 768/81	30-04-84
		ZA A 8101972	24-11-82
US A 5426036	20-06-95	AT E 142263	15-09-96
		AU A1 75154/91	24-10-91
		AU B2 630287	22-10-92
		BR A 9101587	10-12-91
		CA AA 2040810	22-10-91
		CN A 1055952	06-11-91
		CZ A3 9101101	11-08-93
		DE A1 4012818	24-10-91
		DE C0 59108128	10-10-96
		DK T3 453969	10-02-97
		EP A1 453969	30-10-91
		EP B1 453969	04-09-96
		ES T3 2093043	16-12-96
		FI A0 911882	18-04-91
		FI A 911882	22-10-91
		GR T3 3021040	31-12-96
		HR A1 940770	30-06-97
		HU A0 911302	28-10-91
		HU A2 57268	28-11-91
		HU B 210358	28-04-95
		IL A0 97903	21-06-92
		JP A2 4228086	18-08-92